10-1-1995

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JOURNAL OF BACTERIOLOGY, Oct. 1995, p. 6001–6004 Vol. 177, No. 20
0021-9193/95/$04.00+0

Synergistic Induction of the Heat Shock Response in *Escherichia coli* by Simultaneous Treatment with Chemical Inducers

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Received 20 March 1995/Accepted 8 August 1995

***Escherichia coli* strains carrying transcriptional fusions of four σ^{32}-controlled *E. coli* heat shock promoters to *luxCDABE* or *lacZ* reporter genes were stressed by chemicals added singly or in pairs. Much more than additive induction resulted from combinations of cadmium chloride, copper sulfate, ethanol, formamide, 4-nitrophenol, and pentachlorophenol.**

Exposure of cells to sudden increases in temperature or other environmental stresses triggers increased expression of a set of proteins. This response, found in all cells, is known as the heat shock response. The spectrum of inducing agents varies from organism to organism; however, for any particular organism, the heat shock response can be induced by diverse stresses (9). For *Escherichia coli*, these include viral infection, antibiotics, methylating and alkylating agents, hydrogen peroxide (12), and various pollutant molecules (1, 17). The alternative sigma factor σ^{32} coordinately controls induction of about 20 heat shock genes in *E. coli* (5, 20).

The *Vibrio fischeri luxCDABE* genes have been used as a reporter of transcription initiated from the promoter regions of the *E. coli* heat shock genes *dnaK*, *grpE* (17), and *lon* (this study). *E. coli* strains containing these fusions respond to the presence of a variety of chemicals by increasing bioluminescence and are useful to detect sublethal doses of environmental insults (16–18). It was of interest to determine how these biosensor strains would respond to combinations of chemicals, because environmental samples may contain mixtures of toxicants.

Plasmids, strains, and bioluminescence measurement. *E. coli* MC4100 lysogenic for λpF13-(*PrpoD*$_{hs}$-*lacZ*) (19), WM1202 containing pRY002 (*dnaKp*::*lux*), and TV1061 containing pGrpELux5 (17) have been described. *E. coli* DPD1006 containing the *lon′*::*lux* fusion plasmid, pLonLux2, in host RFM443 (10) was constructed as previously described (17), by using these primers for PCR amplification: 5′-ACTTAAGGATCCAAGC GATGGCGCGTAAAA-3′ and 5′-AGCAGCGAATTCATC GCCGCTTCCAGACAA-3′. These primers include, respectively, nucleotides −308 to −290 and +203 to +184 relative to the start point of *lon* transcription (2). The DNA sequences of the promotor regions of pLonLux2 and pRY002 were determined in one direction by a method previously used to verify the sequence of the *grpE* promoter region of pGrpELux5 (18). The sequences determined for pLonLux2 and pRY002 were identical to the promoter region sequences of *lon* (2) and *dnaK* (3), respectively. Bioluminescence was quantitated in a microtiter plate luminometer with log-phase cells in LB medium (11), as previously described (17).

Synergistic increases in bioluminescence from a *lon′*::*lux* gene fusion. Two chemical inducers of the heat shock response, pentachlorophenol and ethanol, were added singly and in combination to *E. coli* DPD1006. Figure 1A shows the bioluminescence at 26°C as a function of time. The combination of inducers as well as each individual inducer resulted in transient increases in light output, as previously observed with other strains containing heat shock promoter-*lux* fusions (16, 17). Specific induction units (SIU), representing the increased light output due to the presence of the inducer, normalized to 10^7 cells, were calculated by using the formula SIU = ($RLU_x - RLU_c$)/10^7 cells, where RLU_x is the bioluminescence of treated cells, RLU_c is the bioluminescence of untreated cells, and the cell density is calculated from the Klett measurement recorded at the beginning of the experiment and the conversion factor of 5.6×10^6 cells ml^{-1} Klett $unit^{-1}$, determined from serial dilution and plating of five independent cultures grown at 26°C to mid-exponential phase. Treatment of strain DPD1006 with pentachlorophenol for 60 min yielded 0.53 SIU; the addition of ethanol yielded 18.0 SIU. The combination yielded 45.1 SIU, which is substantially greater than the sum of the SIU for the individual compounds. Synergistic actions of ethanol and pentachlorophenol were also observed with several other concentrations of pentachlorophenol in the presence of ethanol (Fig. 1B). These more than additive responses suggested synergistic action of pentachlorophenol and ethanol on induction of the heat shock response in *E. coli.*

The combination of pentachlorophenol and ethanol does not increase plasmid levels. To investigate if changes in plasmid level were a factor in the bioluminescent response observed, *E. coli* DPD1006 was grown in LB medium at 26°C and treated with 3% (vol/vol) ethanol, 37.5 μg of pentachlorophenol per ml, or a combination of the agents. Light production from 100-μl samples, culture turbidity, and plasmid content were determined 60 min after addition of the chemicals. Similar to the results from microtiter plate induction experiments, the bioluminescence from the cultures treated with either pentachlorophenol (3.83 RLU) or ethanol (21.0 RLU) was greater than that from the untreated cells (0.01 RLU). The combination of pentachlorophenol and ethanol resulted in a level of light production that was significantly greater than additive (40.32 RLU). The plasmid content was determined by densitometry of plasmid DNA isolated by alkaline lysis, linearized with the restriction enzyme *Eco*RI, separated by agarose gel electrophoresis, and stained with ethidium bromide. Untreated cells were found to have 1.8×10^{-6} ng of plasmid per cell, while the cells treated with pentachlorophenol and ethanol

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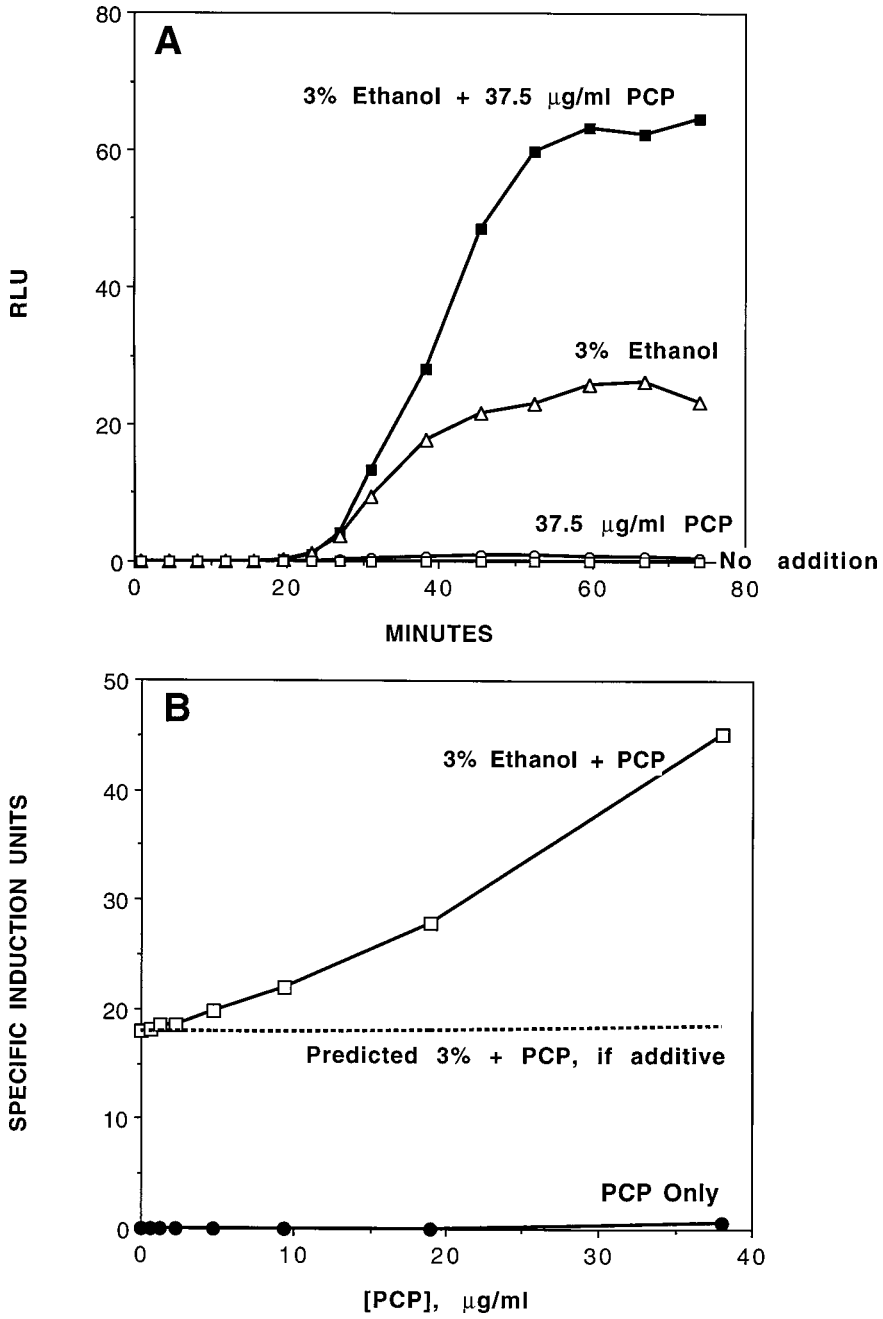

FIG. 1. Synergistic induction of a *lon'*::*lux* fusion by the combination of 37.5 μg of pentachlorophenol (PCP) per ml and 3% (vol/vol) ethanol. (A) Kinetics of light production. (B) SIU at various concentrations of pentachlorophenol. *E. coli* DPD1006 was grown and tested at 26°C in LB medium. SIU were calculated at 60 min after addition of the chemicals to the cells as described in the text.

contained 1.6×10^{-6} ng of plasmid per cell. Thus, the increases in bioluminescence upon chemical treatment were unlikely due to increases in plasmid levels.

Synergistic induction from a chromosomal *PrpoD*$_{hs}$*-lacZ* fusion. The effects of combined inducers on a strain with a different reporter of heat shock gene expression was tested with *E. coli* MC4100 lysogenic for λpF13-(*PrpoD*$_{hs}$*-lacZ*). Following a 31-min chemical treatment of cells grown in LB medium at 30°C, β-galactosidase activity was assayed with the Galacto-Light chemiluminescent system (Tropix, Inc.) by a modification of the procedure suggested by the vendor. Chloroform (20 μl) was added to the lysis solution (80 μl) for cell permeabilization. After incubation with the chemiluminescent substrate at 26°C, Emerald luminescence enhancer was injected and chemiluminescence was quantitated by using a Dynatech ML3000 luminometer in the enhanced-flash mode. Means of quadruplicate samples are reported. The specific β-galactosidase activity was calculated by normalizing the activity to the cell concentration with the units RLU min^{-1} 10^9 $cells^{-1}$, where RLU is the light measured, minutes are the duration of the β-galactosidase assay reaction, and cell density is calculated on the basis of the turbidity measurement. The

TABLE 1. Effects of combinations of inducers on the *grpE′*::*lux* genetic fusion[a]

First compound and concn	SIU for first compound	Second compound and SIU for both	Predicted SIU (if additive)[b]
$CdCl_2$ (mM)		$CuSO_4$ (2.0 mM)	
0	0.00	0.46	
0.016	−0.07	**1.21**	**0.39**
0.031	−0.07	**1.57**	**0.39**
0.062	−0.03	**2.01**	**0.43**
0.125	0.20	**2.21**	**0.66**
0.250	0.22	**0.95**	**0.70**
0.500	−0.55	−0.67	
1.000	−0.82	−0.85	
$CdCl_2$ (mM)		Ethanol (2%)	
0	0.00	31.99	
0.062	0.03	**40.40**	**32.02**
0.125	0.36	**40.38**	**32.34**
0.25	0.51	31.56	32.49
0.5	0.09	6.93	
1	−0.52	0.68	
$CuSO_4$ (mM)		Pentachlorophenol (37.5 μg/ml)	
0	0.06	0.45	
0.08	0.01	**0.63**	**0.46**
0.16	0.02	**0.74**	**0.46**
0.31	0.04	**0.91**	**0.48**
0.63	0.06	**1.40**	**0.50**
1.25	0.10	**1.62**	**0.55**
2.50	1.34	**8.49**	**1.79**
5.00	13.15	−0.13	13.59
Formamide (%)		Ethanol (4%)	
0	0.00	69.65	
0.06	0.24	**99.60**	**69.83**
0.13	0.33	**102.16**	**70.01**
0.25	0.52	**106.56**	**70.19**
0.50	0.84	**120.83**	**70.55**
1.00	1.47	**125.68**	**71.08**
2.00	2.24	**84.34**	**71.80**
4.00	1.23	2.28	70.90
4-Nitrophenol (μg/ml)		$CuSO_4$ (3.0 mM)	
0	0.00	0.31	
1.60	0.00	**0.41**	**0.31**
3.10	0.01	**0.57**	**0.32**
6.25	0.03	**1.00**	**0.34**
12.50	0.08	**1.84**	**0.39**
25.00	0.26	**4.61**	**0.57**
50.00	1.22	0.13	1.52
100.00	0.89	−0.06	1.20
Pentachlorophenol (μg/ml)		Ethanol (2%)	
0	0.02	11.43	
0.6	0.08	12.09	11.51
1.2	0.19	12.47	11.62
2.3	0.13	12.65	11.56
4.7	0.27	13.18	11.70
9.4	0.40	**14.26**	**11.83**
19	0.63	**17.73**	**12.06**
38	1.34	**29.90**	**12.77**

[a] Cadmium chloride (99.995%; Johnson Matthey) was diluted into LB medium from a 100 mM stock solution in water. Copper(II) sulfate pentahydrate (Fisher) was diluted from a 250 mM stock solution in water. Pentachlorophenol (99%; Aldrich) was diluted from a 100-mg/ml solution in ethanol; the final concentration of ethanol after the dilution did not induce a stress response. 4-Nitrophenol (spectophotometric grade; Sigma) was diluted from a 400-mg/ml solution in methanol; the final concentration of methanol after the dilution did not induce a stress response. Ethanol (200 proof; Punctilious; Quantum Chemical Corp.) and formamide (molecular biology grade, deionized with a mixed bed resin; Gibco BRL) were added directly to the LB medium. SIU calculations were made at the same time point for a given experiment. Typically, the calculation was done at 60 min after the addition of chemicals to cells, unless a peak in RLU readings was present prior to 60 min, in which case the calculations were done at time the peak occurred.

[b] Combinations in boldface are those for which the observed SIU were 1.2-fold greater or more than the predicted SIU or for which the observed SIU were 2.0 SIU greater or more than the predicted SIU.

increase in β-galactosidase activity over the control for pentachlorophenol treatment was 5.1 RLU min^{-1} 10^9 $cells^{-1}$, the increase for ethanol treatment was 4.7 RLU min^{-1} 10^9 $cells^{-1}$, and the increase for the combination was 24.0 RLU min^{-1} 10^9 $cells^{-1}$, which is greater than expected if the responses were additive. Thus, synergistic induction was observed with a single-copy fusion to a *lacZ* reporter, as well as with a multiple-copy fusion to a *lux* reporter.

Synergistic induction from many combinations of chemicals. The generality of the synergistic induction of the heat shock response was addressed by testing the effects of numerous combinations of chemical inducers on the three *lux* gene fusions. Table 1 shows data for combinations tested with the *grpE′*::*lux* fusion strain, TV1061. Similar results were also observed for the *lon′*::*lux* fusion strain, DPD1006, and the *dnaKp*::*lux* fusion strain, WM1202 (data not shown). For all three strains, the combinations of cadmium chloride and ethanol, pentachlorophenol and ethanol, pentachlorophenol and copper sulfate, and 4-nitrophenol and copper sulfate resulted in more than additive induction. Furthermore, the combination of cadmium chloride and copper sulfate resulted in more than additive induction of the *grpE′*::*lux* and the *lon′*::*lux* fusion-containing strains, as did the combination of formamide and ethanol for the *grpE′*::*lux* fusion strain. Interestingly, the induction synergy was observed with combinations in which one chemical did not yield an induction. Addition of cadmium chloride alone did not induce increased bioluminescence from the *dnaKp*::*lux* fusion strain. In contrast, simultaneous additions of 4% ethanol and cadmium chloride yielded much greater induction than did this concentration of ethanol alone (Fig. 2).

The increased bioluminescence represents the increased transcription due to the presence of the chemical if the reporter functions are unaffected by the inducer. This assumption was incorrect when the concentrations of compounds approached toxic levels, and decreased bioluminescence resulted (Table 1). This decrease may be a balance between induction of *lux* transcription and toxic effects upon the cell, which result in limitation of cellular metabolism required for bioluminescence or the function of any of the five *lux* gene products. Despite this potential underestimation, numerous examples of combinations of chemicals resulting in synergistic induction were observed, suggesting that the nonadditivity is a general phenomenon in *E. coli.*

Synergistic heat shock protein induction also appears to be conserved in several phyla. There are examples of such synergy in mammalian (6, 8, 13–15), fungal (4), and amphibian (7) cells. In four cases, this synergy is at the level of mRNA synthesis (4, 7, 14, 15), suggesting transcriptional control. Our observations with transcriptional fusions are consistent with this phenomenon also occurring at the transcriptional level in bacteria. Synergistic induction thus appears to be another example of the remarkable similarity of the heat shock response in many organisms, which is consistent with its fundamental importance in the response to a variety of stresses.

The synergistic induction of the heat shock response has implications for the application of biosensor strains in environmental stress detection. Environmental samples would likely

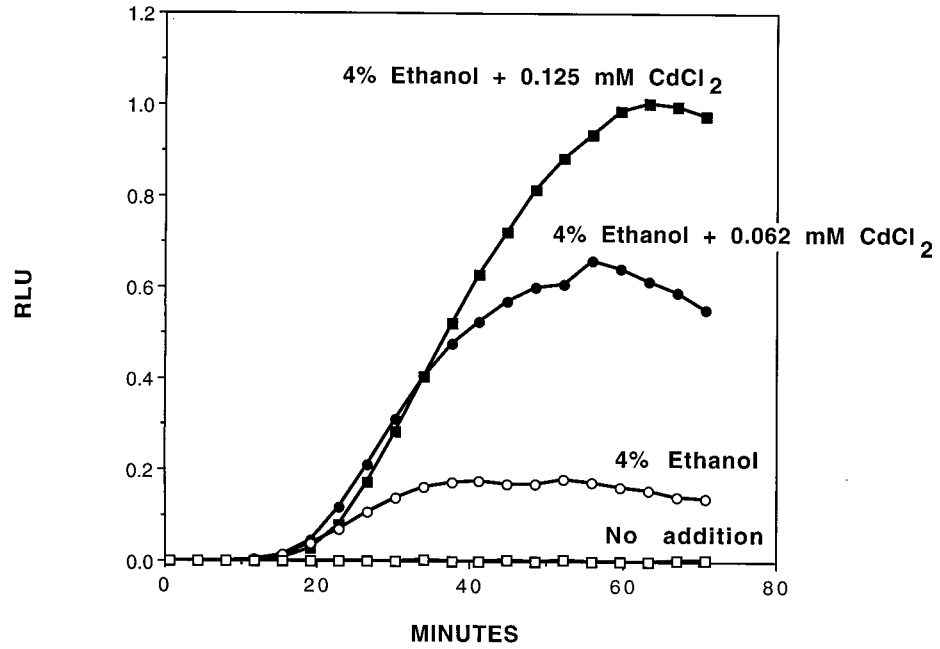

FIG. 2. Cadmium chloride alone does not result in induction of a *dnaKp*::*lux* fusion yet increases induction due to ethanol. Shown are the kinetics of light production in the presence of 4% (vol/vol) ethanol alone or combined with 0.062 or 0.125 mM cadmium chloride. *E. coli* WM1202 was grown and tested at 26°C in LB medium.

contain many components. The toxicity of such combinations would be difficult to predict. Use of strains containing heat shock promoter-*lux* fusions would allow detection of sublethal yet stress-producing environmental conditions. These results suggest that in many cases, multiple toxicants, rather than dampening induction, may enhance it. Furthermore, it may be useful to deliberately add a synergist to expand the range of concentrations and compounds detected.

We thank our DuPont colleagues William Majarian and Roslyn Young for sharing their initial observations that the combination of $CdCl_2$ and ethanol induced the *dnaKp*::*lux* fusion-containing strains, Rolf Joerger and Ed Hendrickson for suggestions on plasmid content determination, Mary Bailey for oligonucleotide synthesis, Shimshon Belkin and Dana Smulski for discussions of this research as it was in progress, and Neil Feltham and Carol Perrotto for literature searches. We also gratefully acknowledge the gift of *E. coli* strains from Takashi Yura.

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